Studies on the Phenomenon of High Promotion by Nucleic Acid of the Production of Streptolysin-S of Hemolytic Streptococcus.

Part 21.

A Simplified Method for Isolating High Potency Streptolysin-S Preparation*

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Since the finding [1] of streptolysin-S formation promoting effect of ribonucleic acid, a number of methods have been developed, whereby partially purified streptolysin-S preparations with high hemolytic potencies could be obtained [for summaries see Bernheimer,[2] 1954, Okamoto,[3] 1962, and reference [4]].

The preceding paper [5] includes reports on the isolation of partially purified streptolysin-S preparation with minimum hemolytic concentrations of 1:100~200 millions from the supernatant fluid of 30-hour culture of *Streptococcus hemolyticus* grown on 1% RNase-Core (AF) broth.

Shortly afterward, it was observed that highly hemolytic supernatant could be obtained when the cocci was grown on broth, to which both yeast ribonucleic acid and pancreatic ribonuclease (RNase 1) had been added.

Employing the information disclosed by some preliminary experiments, a method has been developed for isolating streptolysin-S preparation having minimum hemolytic concentrations of 1:100~200 millions fom 30-hour culture fluid of the cocci grown on broth containing 1% commercial yeast ribonucleate and 1 mg of RNase I per 100 ml broth.

In the present communication, the result of such experiments will be described in brief.

MATERIALS AND METHODS

Strain: A stock laboratory strain of *Streptococcus hemolyticus* "S" was used.

Ribonucleic acid: Commercial yeast sodium nucleate "Merck" was used through-

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out.

RNase I solution: Two mg of finely pulverized pancreatic ribonuclease (SIGMA CHEMICAL COMPANY, 5X crystallized) was suspended in 2 ml of 70% alcohol, and placed, with occasional shaking, at room temperature for 3 hours to effect sterilization.

10% yeast RNA solution: Ten gm of yeast sodium ribonucleate "Merck" was dissolved in 90 ml of distilled water (the solution was neutralized with Na_2CO_3 solution, if necessary), and sterilized at 100°C for 30 minutes.

Preparation of broth: Peptone-infusion broth, pH 7.6, was prepared as described previously (1,000 ml water, 500 gm beef muscle, 10 gm peptone and 5 gm NaCl).

Broth containing yeast RNA and RNase I: Immediately before inoculation with the cocci, 10 ml of 10% yeast RNA solution and 1 ml of RNase I solution were added to 100 ml of peptone-infusion broth, pH 7.6.

Estimation of hemolytic activity: The serial dilution method was used. The hemolytic activity was expressed by the minimum hemolytic concentration, on a weight basis, of the partially purified streptolysin-S preparations.

EXPERIMENTAL

110 ml of broth containing 1 gm of yeast sodium ribonucleate and 1 mg of RNase I was inoculated with 1 ml of 18-hour broth culure of *Streptococcus hemolyticus*, and incubated at 37°C. After 30 hours, the culture was chilled, and centrifuged at 3,500 r.p.m. for 20 minutes. The clear supernatant was tested to be hemolytic up to dilutions of one in 256,000 (~512,000).

The supernatant was then subjected to the isolation experiment of streptolysin-S. The whole isolation experiment was carried out in a cold-room (±2°C).

As shown in Table I, the procedure comprises of two courses:

I. Isolation of R-AI Fraction

To the supernatant was added 5 ml of 28% ammonia, and the precipitate formed was removed by centrifugation. To the clear supernatant fluid thus obtained, after bringing the pH to 6 with glacial acetic acid, was added 1.2 volumes (120 ml) of ice-cold ethanol to effect precipitation. The precipitate was collected by centrifugation, and washed twice with alcohol and once with ether, and finally placed *in vacuo*. A greyish-white product, designated R-AI Fraction, weighed 180 mg. The fraction was found to be hemolytic in a weight-volume ratio of 1 to 102,400,000.

II. Isolation of R-INF_1 Fraction

100 mg of the R-AI Fraction was dissolved in 5 ml of distilled water. The 2% R-AI Fraction solution, after neutralization with 10% Na_2CO_3, was centrifuged to remove some brownish-colored insoluble material. To the supernatant was added 0.05 ml of glacial acetic acid, and then centrifuged. To the clear supernatant thus obtained, after adjusting pH at 6 with 10% Na_2CO_3, was added 1.2 volumes of ice-cold ethanol, and after standing for 15 minutes in ice-water, the precipitate formed was collected by centrifugation, and treated with ethanol and ether, and dried *in vacuo*. The product, designated R-INF_1 Fraction, was white or greyish-white in color. Yield, 43 mg (corresponds to 43% of the R-AI Fraction). The R-INF_1 Fraction was fouud to be hemolytic in a weight-volume ratio of 1 to 102,400,000～204,800,000.

SUMMARY

A very simplified method was described, whereby a high potency streptolysin-S preparation (minimum hemolytic concentration=1:100～200 millions) from culture fluid of *Streptocooous hemolyticus* grown on 1% commercial yeast nucleic-acid broth containing 1 mg RNase I per 100 ml broth.

The main advantage of the present method over the method of isolation of high potency streptolysin-S preparation from culture fluid of hemolytic streptococci grown on 1% Core II (or I)-broth is that commercial yeast nucleic acid can be directly, but together with a minute amount of pancreatic ribonuclease, used for the preparation of culture medium. It is therefore not necessary to separate Core (AF) from the ribonuclease digest of yeast ribonucleic acid before the preparation of the culture medium.

REFERENCES

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Table 1. Isolation of streptolysin-S preparation from culture fluid of *Streptococcus hemolyicus* grown on 1% yeast ribonucleic acid broth containing pancreatic ribonuclease

I. Course :

110 ml of 30-hour culture fluid of *Streptococcus hemolyticus* grown on 1% yeast ribonucleic-acid broth containing 1 mg RNase I per 100 ml broth

Chilled, centrifuged (below 4°C at 3,500 r.p.m. for 15′).

Sediment
B. Fraction, 30 mg

Supernatant [1:256,000]

After addition of 5 ml 28% ammonia, centrifuged.

Sediment
P. Fraction, 84 mg
(1:16,000)

Supernatant

After adjusting pH at 6 by addition of glacial acetic acid, 1.2 volumes (120 ml) of ice-cold ethanol was added, and then centrifuged.

Sediment
*R-AI Fraction, 180 mg
(1:102,400,000)

Supernatant

After addition of 80 ml of ice-cold ethanol, centrifuged.

Sediment
R-AII Fraction, 192 mg
(1:32,000)

Supernatant (discarded)

Each fraction was washed with ethanol and ether, and then preserved in a vacuum desiccator.

*R indicates ribonuclease.

( ) = The hemolytic activity as expressed by minimum hemolytic concentration of a streptolysin-S sample on a weight basis.

II. Course :

100 mg of R-AI Fraction (1:102,400,000) dissolved in 5 ml of distilled water

After neutralization at pH 7 with 10% Na_2CO_3, centrifuged (below 4°C at 3,500 r.p.m. for 15′).

Sediment
(discarded)

Supernatant

After addition of 0.05 ml glacial acetic acid, centrifuged.

Sediment
(discarded)

Supernatant

After adjusting pH at 6 with 10% Na_2CO_3, 1.2 volumes (6 ml) of ice-cold ethanol was added, centrifuged.

Sediment
$R\text{-}INF_1$ Fraction, 43 mg
(1:204,800,000)

Supernatant

After addition of 4 ml of ice-cold ethanol, centrifuged.

Sediment
$R\text{-}INF_2$ Fraction, 13 mg
(1:3,200,000)

Supernatant (discarded)